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AD_____

GRANT NUMBER DAMD17-96-1-6102

TITLE: TGF-alpha Expression During Breast Tumorigenesis

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Cincinnati, Ohio 45267-0529

REPORT DATE: July 1998

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE		Form Approved OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE July 1998	3. REPORT TYPE AND DATES COVERED Annual (1 Jul 97 - 30 Jun 98)	

4. TITLE AND SUBTITLE TGF-alpha Expression During Breast Tumorigenesis

5. FUNDING NUMBERS
DAMD17-96-1-6102

6. AUTHOR(S)
Kevin Taylor

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Cincinnati
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

19981210 089

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

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12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Transforming Growth Factor-alpha (TGFα) is an oncogenic growth factor that drives estrogen-independent breast tumor proliferation via an autocrine loop. It's receptor, Epidermal Growth Factor Receptor (EGFR), is a proto-oncogene that may be overexpressed in some breast tumors. Normal breast TGFα and EGFR are expressed in an estrogen-dependent cell specific manner. To determine whether these oncogenes are important in the early stages of tumorigenesis, we compared the TGFα mRNA and protein expression in normal epithelium and tumor cell lines and examined the expression of TGFα and EGFR mRNA and protein expression in normal epithelium, proliferative breast disease, and carcinoma in situ. Breast cancer cell lines expressed primarily the expected 4.6 kb message. No evidence of splice variants was detected. Interestingly, protein expression from freshly isolated normal and tumor epithelium expressed primarily the soluble form of TGFα while the cell lines expressed the transmembrane form. This suggests that tissue culture may affect the normal regulation of this protein. TGFα protein expression in archival human tissues was constant during progression from benign to invasive disease. However, EGFR expression increased at the stage of proliferative breast disease, suggesting that increased EGFR expression is an important early event in the progression towards invasive disease.

14. SUBJECT TERMS Breast Cancer EGFR Breast Tumorigenesis TGF-alpha oncogenes autocrine/paracrine			15. NUMBER OF PAGES 24
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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INTRODUCTION

<u>Breast Cancer As a Process.</u> The etiology of breast cancer can be defined in terms of the genetic changes which predispose breast epithelium to disregulated growth. These genetic changes may be inherited or the result of environmental insults. Regardless of the mechanism, these genetic changes result in the increased expression of oncogenes and the loss of expression of tumor suppressor genes, leading to uncontrolled proliferation (1-3). Endogenous estrogens are among the environmental influences which contribute to breast tumor development. Estrogens play a significant role in the normal growth and development of the breast (4). However, there is now little doubt that estrogenic stimulation of normal breast epithelial proliferation is central to the etiology of breast cancer development (5). Without estrogen, women do not get breast cancer. The mechanism by which estrogen-induced proliferation predisposes to specific genetic changes is not known. Furthermore, the mechanism of estrogen interaction with proto-oncogene products in normal breast tissue has not been extensively examined. This project focuses on the interaction of estrogen and one such proto-oncogene, transforming growth factor α (TGFα), during tumor development.

<u>TGFα in Breast Tumorigenesis.</u> Transforming Growth Factor α is commonly expressed at high levels in invasive breast cancer (6-8). This protein is a dominant oncogene, i.e. when overexpressed in breast epithelium, it produces a fully transformed cell (7-11). However, TGFα is also expressed at high levels in normal breast epithelium, and its synthesis is driven by estrogen (12-17). The function of TGFα in normal epithelium, therefore, appears to be quite different than that in tumor cells. Explaining this dichotomy is one of our goals.

Many oncogenes encode growth factors, therefore the effects of growth factors in malignant cells have been well characterized (18). For instance, we know that the effects of growth factors (including TGFα) are dependent on the differentiated state of the cell, that is a growth factor which causes proliferation at one state of cellular differentiation may induce quiescence in a cell in another state (19). TGFα is expressed in normal epithelium, during pre-malignant proliferation, and in both in situ and invasive carcinoma (8, 20-21). Yet its function is clearly

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different over this progression to tumor formation. Understanding the role TGFα plays in normal and premalignant breast tissue is critical to understanding the mechanism through which TGFα mediates its effects in tumors.

Breast Histology. To understand the function of TGFα in the breast, one must understand normal breast histology. Breast epithelium is composed of several epithelial cell types (22). Ducts are lined by luminal and myoepithelial cells both of which are derived from the same stem cell (23-25). The milk-forming unit, or the lobule of the breast, is also composed of two epithelial cell types, alveolar cells and myoepithelial cells (22). The lobular myoepithelial cells are morphologically similar to the ductal myoepithelial cells; however, biochemically they display some differences (our observations). For the most part, myoepithelial differentiation is rare in invasive carcinoma and when ducts are filled with carcinoma at a stage called "in situ carcinoma", the myoepithelial cells are lost, or at best, greatly reduced in number (23). This is true regardless of whether the carcinoma has a more ductal or lobular phenotype. In either case, the cell of origin is thought to reside in the terminal ducto-lobular unit. Hormone-sensitive connective tissue composed of fibroblasts and myofibroblasts surrounds the epithelial cells of the breast. The role these cells play in the process of carcinogenesis is completely unknown. Each of these cell types expresses proto-oncogenes during the normal development and maturation of the breast. The regulation of this expression is the process which goes awry in tumorigenesis. To understand this regulation, we must examine the cell-specific expression of these proto-oncogenes and define the characteristics of normal breast which participate in this regulation.

Our preliminary data suggests that TGFα and its receptor (epidermal growth factor receptor or EGFR) function by two different mechanisms in normal breast. Here we show that the luminal cells of normal, quiescent breast epithelium express TGFα, but not EGFR; therefore, unlike tumor cells, the luminal cells can not respond to secreted TGFα in an autocrine fashion. Myoepithelial cells and adjacent stromal cells express EGFR normally; therefore, in normal breast the target for TGFα production is the myoepithelium and connective tissue. The cellular responses to this

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stimulation by TGFα are not known. Between quiescent epithelium and invasive tumor lie intermediate states of proliferation/transformation. Proliferative breast diseases of various morphologic types carry an increased risk of carcinoma and are considered "premalignant" phenotypes (27). In the literature and in our own experience, luminal EGFR expression is associated with some proliferative and cystic breast diseases (28). In particular, luminal cells with EGFR also express the estrogen receptor (ER) (28). Thus, alterations in EGFR synthesis may appear very early in proliferating breast, driven by estrogen. In fact, antisense studies with TGFα suggest that induction of TGFα synthesis may be the main mechanism for estrogen-induced proliferation in this cell type (15). By the time breast epithelium has the characteristics of in situ carcinoma, TGFα and EGFR clearly co-localize in the luminal cells. The luminal cells up regulate EGFR and in the fully transformed state, respond to TGFα in an autocrine loop. At this stage there is an inverse correlation between EGFR and ER expression, consistent with the concept that over-stimulation of the TGFα/EGFR pathway leads to estrogen-independent growth (29). Some have suggested that cell transformation by EGFR is the result of a "threshold" level of EGFR expression (10). In this case, one would expect quantitatively increased expression in in situ carcinoma or invasive disease relative to these "premalignant" lesions. While the luminal cell is being driven to proliferate, the myoepithelial cell is lost during the transition from premalignant proliferative growth to in situ carcinoma. The mechanism by which these cells are lost, and the consequences of their loss are not known.

In this project we explore the role of TGFα in normal and proliferative breast tissue. Understanding the normal role of TGFα in tumor production is critical for the following reasons. First, TGFα is a major estrogen-responsive gene (33); therefore, to understand estrogen-promotion of tumorigenesis, one must know the consequence of estrogen-induced gene expression. Second, TGFα function changes during the process of tumorigenesis. In normal tissue TGFα operates in a paracrine fashion and may be critical for maintaining normal lobular growth. In fact, there is now evidence that lactation is regulated by TGFα (34). However, in tumors TGFα leads to estrogen-independent growth (9). The effects of TGFα in the steps in between, from normal

cell growth to invasive disease, have not been studied. Finally, current experimental therapies target various points in the TGFα signal transduction pathway. For instance, TGFα-EGFR stimulates cells through the MAP kinase pathway (35). This signal transduction pathway involves the proto-oncogene, ras, as well as two kinases which have been well characterized, protein kinase C (PKC) and protein kinase A (PKA) (36). The action of PKC is of particular note, since when EGFR is activated, it results in PKC activation (37-39). As part of an inhibitory feedback loop, PKC then phosphorylates and inhibits the action of EGFR, preventing over stimulation of the cell (40). These are exactly the types of pathways which one would predict are destroyed during the progression to malignancy. Current clinical trials include the use of Bryostatin, an agent which inhibits PKC (41). Given that TGFα can induce proliferation or differentiation, based on the differentiation state of the receptor cell, it is likely that the signaling pathways downstream of EGFR change during tumorigenesis. Therefore, it is critical that we understand the role TGFα is playing in specific breast cells at the time therapies affecting these pathways are instituted.

In this work, we build on our previous work, examining oncogene expression during tumor development and metastasis. Here we shall define the cell-specific expression of TGFα and EGFR in histologically similar premalignant states from women who do or do not progress to invasive tumor. Expression will be correlated with histopathologic types of proliferative breast disease, extent of disease types appearing concurrently, and estrogen receptor status. The observation that TGFα and EGFR must form a paracrine loop between myoepithelial and luminal cells is the result of careful evaluation of benign breast tissues for oncogene expression during a study comparing oncogene expression in benign, invasive, and metastatic tumors (43). In addition, the PI's mentor has collected an extensive set of premalignant breast tissue which are used for these studies. The results of this study will provide an invaluable database of cell-specific gene expression in vivo which can then be correlated with in vitro tissue culture work.

1. Prepare riboprobes for in situ hybridization: subcloning of cDNAs, sequencing of constructs. (Month 0-6).

Conclusion: 925 basepairs of the TGFα gene has been subcloned into the PGEM 3Z plasmid and is in the 5' to 3' orientation relative to the T7 promoter. This goal is completed and presented in 1996-1997 Annual Report.

2. Identify TGFα splice variants in breast cancer cell lines. (Month 6-9).

Initially, the riboprobe was labeled using the Genius Nonradioactive Nucleic Acid Labeling and Detection System (Boehringer-Mannheim Corp., Indianapolis, IN). Due to the poor sensitivity of the digoxigenin-labeled riboprobe labeling was switched from a non-radioactive to a radioactive labeling method. The TGFα probe is now labeled using a standard Pharmacia oligonucleotide labeling protocol (Pharamacia, Piscataway, PA). Seven breast cancer cell lines: Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A (ATCC, Rockville, MD) have been examined and show that in all cases the primary mRNA transcript is at 4.6 kb (Figure 1).

Conclusion: Northern blots of isolated RNA on breast cancer cell lines were performed, and probed with the radiolabeled TGFα oligo-probe. All cell lines express the expected 4.6 kb transcript as quoted in the literature (6, 8, 9, 43). No unusual transcripts which would indicate splice variants were detected.

3. Identify TGFα protein sizes in breast cell lines: Western blot. (Month 6-9).

Conclusion: Protein was isolated from: Hs-578 Bst, SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A cell lines and all primarily express the transmembrane form. This goal is completed and presented in 1996-1997 Annual Report.

4. Repeat 2 and 3 in normal breast cells or organoids. (Month 9-12).

We have examined freshly isolated RNA from eight organoid samples under multiple conditions and have concluded that the TGFα mRNA is too low to detect by Northern blot analysis. Therefore, in order to detect TGFα mRNA expression we are currently developing competitive polymerase chain reaction (PCR).

Previously, Westerns were run on seven breast cancer cell lines, cultured fibroblasts and a fresh breast tissue sample. The fresh breast tissue sample expressed primarily the soluble form (6 kD)of TGFα (reported in 1996-1997 Annual Report). We needed to further investigate if this difference in TGFα protein expression is due to culturing of the cells or an underlying difference between transformed vs. non-transformed epithelial cells. To accomplish this a Western blot was performed on five more patient samples (fresh tissue or isolated organoids) and the cell line MCF-10A. Of the five patient samples one sample was from freshly isolated tumor, and this sample along with the other four normal tissue samples express primarily the soluble form (6 kD) of TGFα (Figure 2). These data indicate that the difference in expression from the fresh tissue samples and cell lines is likely due to culturing of cell lines.

Conclusion: TGFα mRNA from normal epithelium is too low to detect by Northern blot analysis and we are currently in the process of developing competitive PCR to detect message expression. Western blot analysis of patient samples (whole tissue, isolated organoids, and tumor) primarily express the soluble form of TGFα. This is in contrast to cell lines expressing primarily the transmembrane form of the protein, suggesting that fresh tissue may be a more appropriate model to study TGFα protein regulation in breast tissue.

5. Develop splice-specific probes. (Month 9-12).

Conclusion: Northern blot analysis of seven breast cancer cell lines (Figure 1) and fresh breast tissue samples and organoids (Figure 2) using a radiolabeled TGFα oligo-probe do not indicate any potential splice variants.

6. Examine normal and proliferative breast tissue for cell-specific expression of TGFα. (Month 12-24).

Originally most tissues were to be tested for TGFα mRNA expression by in situ hybridization. However, despite good in situ results using cells lines, very inconsistent results were obtained in human archival tissue. To expedite our analysis of TGFα/EGFR expression we identified TGFα expression by immunohistochemistry (IHC). Paraffin embedded formalin fixed tissue from 90 patients were collected and characterized. Protein expression was determined in the luminal epithelial cells and myoepithelial cells of ducts and lobules, as well as the microvasculature, stromal cells and infiltrating lymphocytes. Expression of these proteins in each histological subtype was compared with one another as breast tissue progressed towards invasive disease (i.e. proliferative breast disease, carcinoma in situ (cis), and invasive disease). Our data indicate that TGFα expression does not increase in the luminal epithelial cells of the ducts or lobules as the epithelium progresses through these histopathological stages (Figure 3). In the microvasculature a trend is seen in TGFα upregulation in endothelium adjacent to breast lesions. However, EGFR expression increases in the luminal epithelial cells during the proliferative phase of breast tumorigenesis (p < 0.006) compared to normal epithelium, suggesting that upregulation of EGFR is important early on in the progression towards invasive disease (Figure 3). Subsequent to the proliferative stage of progression, EGFR actually decreases.

Conclusion: Analysis of TGFα and EGFR protein expression by IHC in human archival breast tissue reveals: 1. TGFα protein expression is unaltered in the luminal cells as the epithelium progresses through the histopathological subtypes. 2. TGFα is upregulated in endothelium adjacent to breast lesions. 3. EGFR protein expression increases in the luminal cells during the proliferative phase of breast tumorigenesis. This suggests EGFR is an important early event in the progression towards invasive disease.

7. Examine tissue as above for proliferation, estrogen receptor expression, and apoptosis. (Month 18-24).

Estrogen receptor (ER) and progesterone receptor (PR) protein expression were analyzed in a subset of patients. Although there was no consistent difference in ER/PR expression during disease progression, in general high,

functional ER expression correlated with high TGFα
expression and low or non-functional ER expression
correlated with low TGFα expression (Functional ER
expression is defined as the co-expression of PR). These
results are consistent with in vitro data indicating TGFα
expression can be regulated by an estrogen dependent
mechanism (14-16, 33, 43). IHC using the mib-1 monoclonal
antibody to determine proliferation has been done and the
slide are currently being analyzed. Histological analysis
of the human archival breast tissue does not reveal an
appreciable amount of apoptotic bodies. Therefore, my
thesis committee felt that performing the TUNNELL assay to
detect cells undergoing apoptosis would not be informative.

Conclusion: Functional expression of ER (i.e. ER/PR
positive) correlated with expression levels of TGFα. IHC
using the mib-1 monoclonal antibody to determine
proliferation has been performed on all cases and are
currently being analyzed. Histological analysis of the
human archival breast tissue does not reveal an appreciable
amount of apoptotic bodies.

8. Prepare riboprobes for PKC.   (Month 24-30).
See below.

9. Examine tissues as above for alterations in PKC isozyme
expression. (Month 30-36).
See below.

CONCLUSION

We have subcloned 925 basepairs of the TGFα gene into the
PGEM 3Z plasmid and have made a ^{32}P-labeled probe. Northern
blots hybridized with the radiolabeled TGFα probe
identified the 4.8 kb mRNA transcript as the primary TGFα
message in seven breast cancer cell lines: Hs-578 Bst,
SKBR3, MCF-7, T-47-D, MDA-MB-453, MDA-MB-468, and MCF-10A.
No splice variants were detected, despite extensive
exposure on phosphoimaging plates (Figure 1). Northern
blots on 8 patient samples (fresh tissue or organoids) were
performed; however, no TGFα mRNA was detected. Using the
radiolabeled TGFα probe no mRNA is detected. Further work
will focus on developing competitive PCR analysis for TGFα
to detect mRNA expression.

Previously, protein was isolated from the same seven breast
cancer cell lines (see above) and Westerns performed. The

results indicate that the cell lines express primarily the transmembrane bound form (approximately 22 kD) of TGFα. Western blots performed on patient samples (fresh tissue or organoids) express primarily the soluble form (6 kD) of TGFα. Protein isolated from a tumor specimen also expresses primarily the soluble form of TGFα (Figure 2). These data suggest that the difference in protein expression in the cell lines is due to a tissue culture artefact. Suggesting that fresh tissue may be a more appropriate model to study alterations in TGFα expression during breast tumorigenesis. Immunohistochemical analysis of TGFα/EGFR during progression in individual women indicates that EGFR may play a role in the early proliferative process.

Earlier this year these data were evaluated by my thesis committee who felt that the TGFα data obtained from breast cell lines may not reflect the role of TGFα in the early proliferative process. Therefore, upon their recommendation, I have developed procedures for isolating normal human breast epithelium (organoids) and characterized culture conditions which allow multiple passages of the epithelium in vitro. Furthermore, I have developed methods to characterize the isolated cell types using monoclonal antibodies against keratin 19 (K19) as a luminal marker and keratin 14 (K14) as a myoepithelial marker. Examples of immunohistochemical staining for these proteins in isolated organoids are shown (Figure 4). Having developed these procedures I can now address the following issues: 1. Confirm that TGFα processing differs between in vivo and in vitro by examining TGFα from fresh tissue vs. primary and early passage cultures from the same patient. 2. Determine the role of stromal cells in TGFα protein processing during co-culture of isolated organoids in vitro. 3. Determine whether EGFR expression is altered with conversion from a relatively quiescent organoid to a rapidly proliferating monolayer culture. 4. Investigate the proliferative effect of TGFα and EGF treatment on isolated organoids by immunologic detection of bromo-deoxyuridine (BrDU) incorporation.
Given our findings to date, my committee believes that these experiments will allow me to develop functional assays to assess the role of TGFα/EGFR in breast proliferation and develop models in which ER regulation of EGFR expression can be studied. The final objectives of

this proposal were to evaluate PKC isozyme expression. We
originally planned to use RNA for isozyme detection.
However, over the past two years numerous excellent
isozyme-specific antibodies have been developed and are now
commercially available. Isozyme expression of matched
normal and tumor samples will be analyzed by Western.

MATERIALS & METHODS

Tumor cell culture: Breast tumor cell lines Hs-578 Bst,
SKBR3, MCF-7, A431, T-47-D, MDA-MB-453, MDA-MB-468,and MCF-
10A were obtained from ATCC and cultured according to their
protocols. Cells are passaged weekly using trypsin-EDTA.

Organoid isolation: Breast epithelium (organoids) is
dissected from both mastectomy and reduction mammoplasty
specimens, minced, and enzymatically digested with
collagenase as previously described (45). Briefly, tissue
is obtained within 1 hour of surgery, minced with opposing
scalpels to 1 mm3 pieces, and enzymatically digested for 12
to 18 hours at 37 $^{\circ}$C in a rotary shaker (60 rpm) with 150
U/ml Collagenase Type I (Gibco), 100 U/ml Hyaluronidase
(Gibco) in CDM3 media. CDM3 media consists of DMEM/F12
(1:1), 2.6 ng/ml selenium, 100 ng/ml EGF, 0.1 µg/ml
fibronectin (all from Collaborative Research, Bedford, MA),
3 µg/ml insulin, 25 µg/ml transferrin, 10^{-10} M estradiol, 10^{-6} M hydrocortisone, 10^{-8} M cAMP, 10^{-8} M triiodothyronine, 10^{-4}
M ethanolamine, 10^{-4} M phosphoethanolamine, 0.1% BSA, 10
µg/ml ascorbic acid, 20 µg/ml fetuin (all from Sigma, St.
Loius, MO), and 1X trace element mix (Biofluids, Rockville,
MD). Following digestion, epithelial organoids and
fibroblasts are separated from vascular fragments and from
one another by sedimentation.

TGFα radiolabeled probe. The probe was labeled with ^{32}P-CTP
using a standard oligonucleotide labeling kit (Pharmacia,
Piscataway, PA). Briefly, 25 ng of TGFα plasmid DNA is
labeled with ^{32}P-CTP at 37 $^{\circ}$C for 1 hour in a reaction
mixture containing random hexanucleotide primers and 50U of
Klenow fragment. The labeled probe is then isolated on a
Sepharose G50 spin column (Pharmacia, Piscataway, PA).

Northern Blot Analysis. Total cellular RNA is isolated
using RNAzol (CINNA/BIOTEX) and electrophoresed on
formaldehyde-denatured agarose gels as in (65). RNA is
transferred onto Nytran for hybridization.
Prehybridization of the filters is carried out for 4-18
hours at 37°C in 50% formamide, 5x SSC, 0.1%

polyvinylpyrrolidone, 0.1% Ficoll, 0.1 mg/ml salmon sperm DNA, 20 mM sodium phosphate (pH 6.8), and 0.1% SDS. For hybridization the appropriate 5'-end labeled oligonucleotide or ^{32}P-labelled riboprobe is added and incubated at 37° overnight. The filters are then washed in 2x SSC and 1% SDS twice at room temperature, 2x SSC and 1% SDS twice at 37°C, and 0.2x SSC and 1% SDS for 15 minutes at 37°C.

Western blot: Cells or tissues are homogenized in 20mM Tris-HCl, pH 7.4, 2mM EDTA, 0.5mM EGTA for protein analysis (Bradford assay) and then electrophoresed under reducing conditions on 10% acrylamide SDS-PAGE (Laemmli) and transferred to PVDF membranes (Millipore) by the Towbin method. Membranes from each well are cut for replicate analysis, blocked in 5% non-fat dry milk, 0.5M NaCl, 10 mM Tris-HCl, pH 7.5. Primary antibodies (5-10 µg/ml) are incubated in blocking solution at RT overnight. The membranes are washed and incubated one hour at RT with biotinylated anti-mouse/rabbit Ig (1:100, Sigma), washed, and incubated another hour with Streptavidin-alkaline phosphatase (1:200, Sigma) in 5% non-fat milk, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5. The reaction is developed with BCIP/NBT substrate. Samples are calibrated for equal loading by using antibodies against actin.

Immunohistochemistry: 4µm tissue sections are deparaffinized and stained using the Ventanna 320 automated immunostainer (Ventanna Medical Systems, Tucson, AZ). The TGFα antibody Clone 31G7 (Zymed, San Francisco, CA) is used at a dilution of 1:10. The EGFR antibody Clone 213-4.4 (Oncogene Research Products, Cambridge, MA) is used at a dilution of 1:25. The Ki-67 monoclonal antibody mib-1 (Immunotech, West Brook, ME) is used at a dilution of 1:40. The monoclonal antibodies are pretreated with trypsin on the immunostaining system. Each reaction utilizes a horse-radish peroxidase-conjugated secondaries. Slides are then counterstained with hemotoxylin.

BIBLIOGRAPHY

1. Lee EY-HP, Tumor suppressor genes and their alterations in breast cancer. Sem in Cancer Biology. 6:119-125, 1995.
2. Eufjord JE, Thorlacium S, Steinarsdottir M, Valgardsdottir R, Ogmundsdottir HM, Anamthawat-Jonssoon K. p53 abnormalities and genomic instability

in primary human breast carcinomas. Cancer Res 55:646-651, 1995.

3. van Leeuwen F, Nusse R. Oncogene activation and oncogene cooperation in MMTV-induced mouse mammary cancer. Sem in Cancer Biol. 6:127-133, 1995.

4. Richards JE, Shyamala G, Nandi S. Estrogen receptor in normal and neoplastic mouse mammary tissues. Cancer Res 34:2764-2772, 1974.

5. Dickson RB, Lippman ME. Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocrine Rev. 8:29-43, 1987.

6. Salomon DS, Kim N, Saeki T, Ciardiello F. Transforming growth factor a: an oncodevelopmental growth factor. Cancer Cells 2:389-397, 1990.

7. Madsen MW, Lyddesfeldy AE, Laursen I, Nielsen KV, Briand P. Altered gene expression of c-myc, epidermal growth factor receptor, transforming growth factor-a, and c-erb-B2 in an immortalized human breast epithelial cell line, HMT-3522, is associated with decreased growth factor requirements. Cancer Res. 52:1210-1217, 1992.

8. Perroteau I, Salomon D, DeBortoli M, Kidwell W, Hazarika P, Pardue R, Dedman J, Tam J. Immunological detection and quantitation of alpha transforming growth factors in human breast carcinoma cells. Breast Cancer Res and Treat. 7:201-210, 1986.

9. Herman ME, Katzenellenbogen BS. Alterations in transforming growth factor-a and -b production and cell responsiveness during the progression of MCF-7 human breast cancer cells to estrogen-autonomous growth. Cancer Res 54:5867-5874, 1994.

10. DiMarco E, Pierce JH, Fleming TP, Kraus MH, Mollay CJ, Aaronson SA, Di Fiore PP. Autocrine interaction between TFGa and the EGF-receptor: quantitative requirements for induction of the malignant phenotype. Oncogene 4:831-838, 1989.

11. Ju WD, Velu TJ, Vass WC, Papageorge AG, Lowry DR. Tumorigenic transformation of NIH 3T3 cells by the autocrine synthesis of transforming growth factor a. New Biol. 3:380-388, 1991.

12. Spitzer E, Zschiesche W, Binas B, Grosse R, Erdmann B. EGF and TGFa modulate structural and functional differentiation of the mammary gland from pregnant mice in vitro: possible role of the arachidonic acid pathway. J Cell Biochem. 57:495-508, 1995.

13. Zwiebel JA, Bano M, Nexo E, Salomon DS, Kidwell WR. Partial purification of transforming growth factors from human milk. Cancer Res. 46:933-939, 1986.

14. Liu SC, Sanfilippo B, Perroteau I, Derynck R, Salomon DS, Kidwell WR. Expression of transforming growth factor a (TGFa) in differentiated rat mammary tumors: estrogen induction of TGFa production. Molec Endocrin. 1:683-692, 1987.
15. Kenney NJ, Saeki T, Gottardis M, Kim N, Garcia-Morales P, Martin MB, Normanno N, Ciardiello F, Day A, Cutler ML, Salomon DS. Expression of transforming growth factor a antisense mRNA inhibits the estrogen-induced production of TGFa and estrogen-induced proliferation of estrogen-responsive human breast cancer cells. J Cell Physiol. 156:497-514, 1993.
16. Manni A, Wright C, Gadger B, Bartholomew M, Herlyn M, Mendelsohn, Masui H, Demers L. Role of transforming growth factor-a-related peptides in the autocrine/paracrine control of experimental breast cancer growth in vitro by estradiol, prolactin,and progesterone. Breast Cancer Res and Treat. 15:73-83, 1990.
17. Saeki T, Cristiano A, Lynch MJ, Brattain M, Kim N, Normanno N, Denney N, Ciardiello F, Salomon DS. Regulation by estrogen through the 5'-flanking region of the Transforming Growth Factor a gene. Molec Endocrin. 5:1955-1963, 1991.
18. Druker BJ, Mamon HJ, Roberts TM. Oncogenes, growth factors, and signal transduction. New Engl J Med. 321:1383-1391, 1989.
19. Sauma S, Huang F, Winawer S, Friedman E. Colon goblet cells lose proliferative response to TGFa as they differentiate. Int J Cancer 61:848-853, 1995.
20. Snedeker SM, Brown CF, DiAugustine RP. Espression and functional properties of transforming growth factor a and epidermal growth factor during mouse mammary gland ductal morphogenesis. Proc Natl Acad Sci USA. 88:276-280, 1991.
21. Scala S, Saeki T, Lynch A, Salomon D, Merino MJ, Bates SE. Coexpression of TGFa, epidermal growth factor receptor, and P-gycoprotein in normal and benign diseased breast tissues. Diagnostic Molec Pathol. 4:136-142, 1995.
22. Rudland PS. Histochemical organization and cellular composition of ductal buds in developing human breast: evidence of cytochemical intermediates between epithelial and myoepithelial cells. J Histochem and Cytochem. 39:1471-1484, 1991.
23. Rudland PS, Leinster SJ, Winstanley J, Green B, Atkinson M, Zakhour HD. Immunocytochemical

identification of cell types in benign and malignant
breast diseases: variations in cell markers accompany
the malignant state. J Histochem Cytochem. 41:543-553,
1993.

24. Rudland PS, Barraclough R. Stem cells in mammary gland
    differentiation and cancer. J Cell Sci Suppl 10:95-
    114, 1988.

25. Noguchi S, Motomura K, Inaji H, Imaoka S, Koyama H.
    Clonal analysis of solitary intraductal papilloma of
    the breast by means of polymerase chain reaction. Am J
    Pathol 144:1320-5, 1994.

26. Silverstein MJ, Lewinsky BS, Waisman JR, Gierson ED,
    Colburn WJ, Senofsky GM, Gamagami P. Infiltrating
    lobular carcinoma: is it different from infiltrating
    duct carcinoma? Cancer 73:1673-1677, 1993

27. Bodian CA, Perzin KH, Lattes R, Hoffmann P, Abernathy
    TG. Prognostic singificance of benign proliferative
    breast disease. Cancer 71:3896-3907, 1993.

28. van Agthoven T. Timmermans M, Foekens JA, Dorssers LCJ,
    Henzen-Logmans SC. Differential expression of
    estrogen, progesterone, and epidermal growth factor
    receptors in normal, benign, and malignant human
    breast tissues using dual staining
    immunohistochemistry. Am J Pathol. 144:1238-1246,
    1994.

29. Newby JC, A'Hern RP, Leek RD, Smith IE, Harris AL,
    Dowsett M. Immunohistochemical assay for epidermal
    growth factor receptor on paraffin-embedded sections:
    validation against ligand-binding assay and clinical
    relevance in breast cancer. Br J Cancer 71:1237-1242,
    1995.

30. Halter SA, Dempsey P. Matsui Y. Stokes MK, Graves-Deal
    R, Hogan BLM, Coffey RJ. Distinctive patterns of
    hyperplasia in transgeic mice with mouse mammary tumor
    virus transforming growth factor-a. Am J Pathol.
    140:1131-1146, 1992.

31. Smas CM, Sui HS. Pref-1, a protein containing EGF-like
    repeats, inhibits adipocyte differentitiation. Cell
    73:725-734, 1993.

32. Bani D, Riva A, Bigazzi M, Sacchi TB. Differentiation
    of breast cancer cells in vitro is promoted by the
    concurrent influence of myoepithelial cells and
    relaxin. Br. J. Cancer. 70:900-904, 1994.

33. Bates SE, Danidson NE, Valverius EM, Freter CE, Dickson
    rB, Tam JP, Kudlow JE, Lippman ME, Salomon DS.
    Expression of transforming growth factor a and its
    messenger ribonucleic acid in human breast cancer: its

regulation by estrogen and its possible functional significance. Molec Endocrinol. 2:543555, 1988.

34. Lin CQ, Dempsey PJ, Coffey RJ, Bissell MJ. Extracellular matrix regulates whey adidic protein gene expression by suppression of TGFa in mouse mammary epithelial cells: studies in culture and in transgenic mice. J Cell Biol. 129:1115-1126, 1995.

35. Ahn NG, Seger R, Bratlien RL, Keltz CD, Tonks NK, Krebs EG. Multiple components in an epidermal growth factor-stimulated protein kinase cascade. J Biol Chem. 7:4220-4227, 1991.

36. Buday L, Downward J. Epidermal growth factor regulates p21ras through the formation of a complex of receptor, Grb2, adaptor protein, and Sos nucleotide exchange factor. Cell 73:611-620, 1993.

37. Osborne CK, Hamilton B, Nover M, Ziegler J. Antagonism between epidermal growth factor and phorbol ester tumor promoters in human breast cancer cells. J Clin Invest. 67:943-951, 1981.

38. Fabbro D, Kung W, Roos W, Regazzi R, Eppenberger U. Epidermal growth factor binding and protein kinase C activites in human breast cancer cell lines: possible quantitative relationship. Cancer Res. 46:2720-2725, 1986.

39. Wyss R, Fabbro D, Regazzi R, Borner C, Takahashi A, Eppenberger U. Phorbol ester and epidermal growth factor receptors in human breast cancer. Anticancer Res. 7:721-728, 1987.

40. Schlessinger J. Allosteric regulation of the epidermal growth factor receptor kinase. J Cell Biol. 103:2067-2072, 1986.

41. Blobe GC, Obeid LM, Hannun YA. Regulation of protein kinase C and role in cancer biology. Cancer and Met Rev. 13:411-431, 1994.

42. Lower E, Heffelfinger SC, Miller MA, Fenoglio-Preiser CM. Phosphotyrosine immunoreactivity is a significant prognostic indicator in breast carcinoma patients. Breast Cancer Res Treat., 35:277-282, 1995.

43. Salomon DS, Brandt R, Ciardiello F, Normanno N. Epidermal Growth Factor-related peptides and their receptors in human malignancies. Critical Review in Oncology/Hematology, 19:183-232, 1995.

44. Lan HY, Wei Mu, Yee YY, Nikolic-Patterson DJ, Atkins RC.A simple, reliable, and sensitive method for nonradioactive In Situ Hybridization: Use of Microwave Heating to Improve Hybridization Efficiency and

Preserve Tissue Morphology. The Journal of
Histochemistry and Cytochemistry 44:281-287, 1996.

45. Petersen OW, vanDeurs B. Preservation of Defined
Phenotypic Traits in Short-Term Cultured Human Breast
Carinoma Derived Epithelial Cells. Cancer Research.
47:856-862, 1987.

Figure 1.

Northern blot showing expression of TGFα mRNA expression
using a ^{32}P labeled probe. Lanes are mRNA isolated from
cell line: (a) HS-578-Bst, (b) MDA-MB-453, (c) MDA-MB-
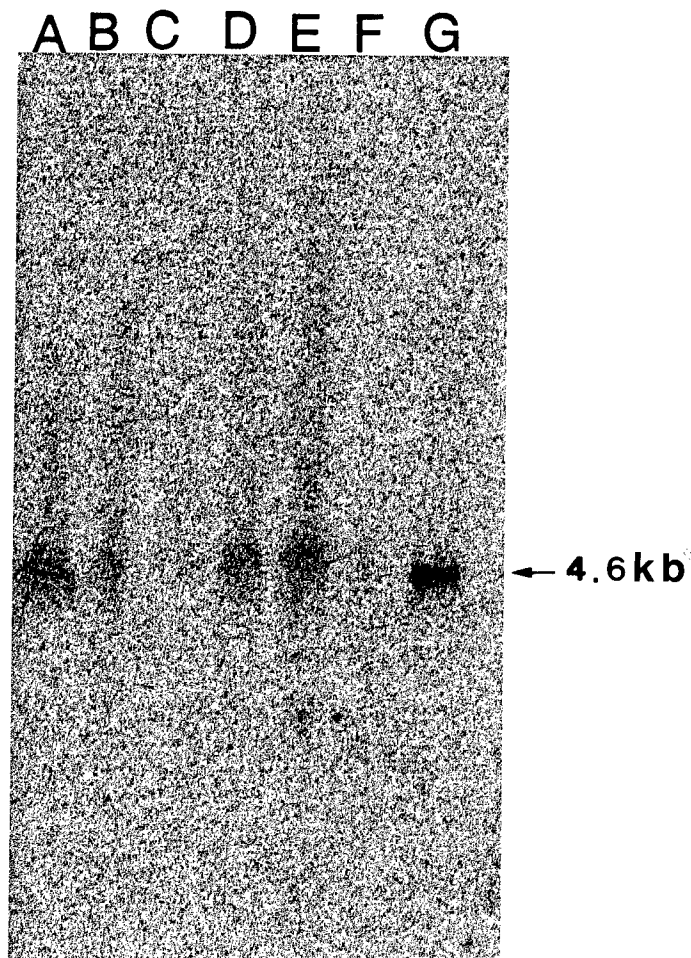468,(d) T-47-D, (e) MCF-7, (f) SKBR3, (g) MCF-10A.

A B C D E F G

← 4.6 kb

Figure 2.

Anti-TGFα Western blot from fresh tissue samples. Lanes are proteins isolated from: (a) MCF-10A cell line, (b) fresh invasive ductal carcinoma, (c) isolated organoids patient A, (d) isolated organoids patient B, (e) fresh breast tissue patient C, (f) fresh breast tissue patient D, (g) commercial 6 kD TGFα.

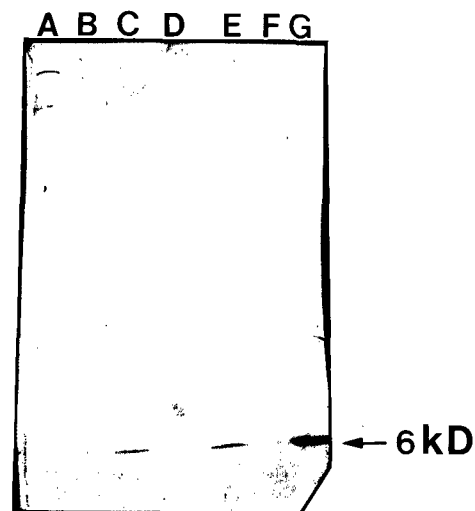Figure 3.

Tables showing (a) EGFR protein expression during tumor progression (* p=0.006). (b) TGFα protein expression in luminal epithelium during tumor progression. Shown are the mean and standard deviation of immunohistochemical stain intensity for each histologic subtype.
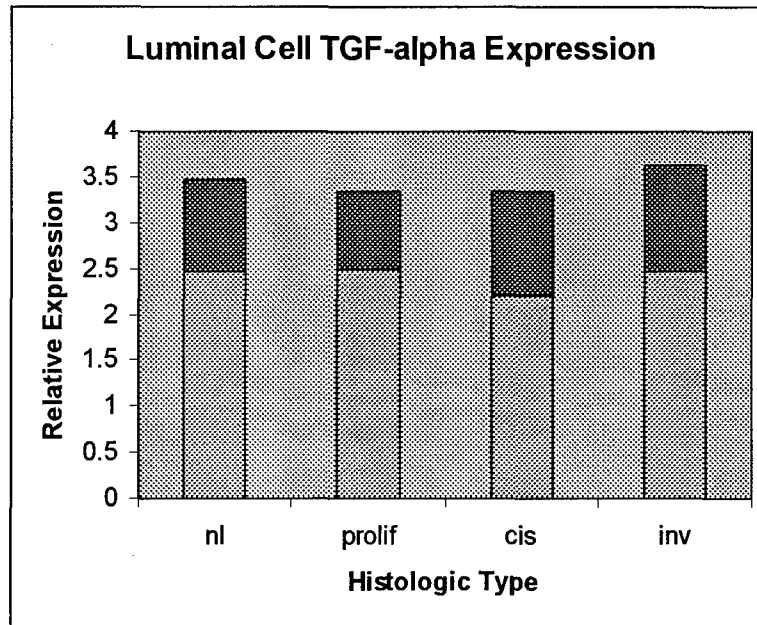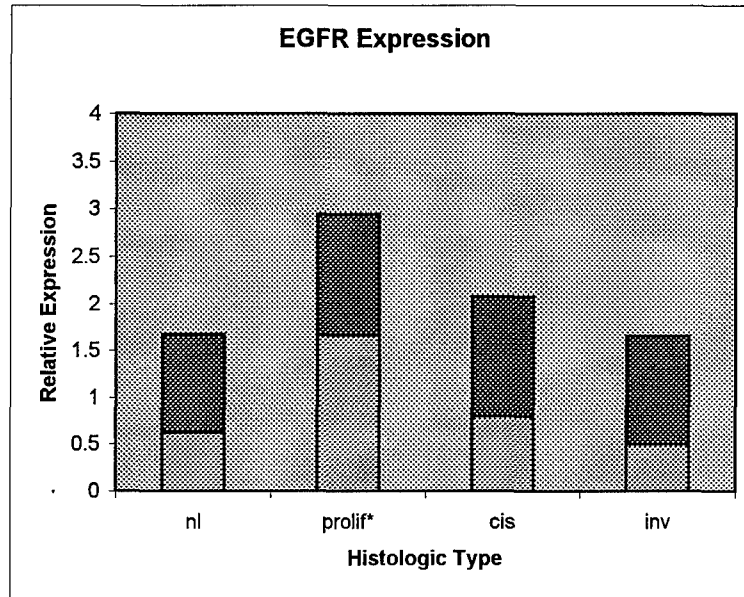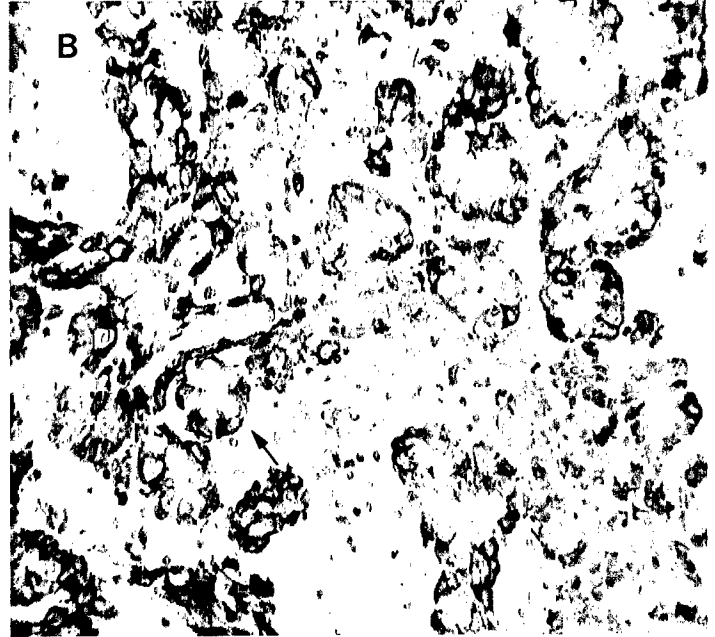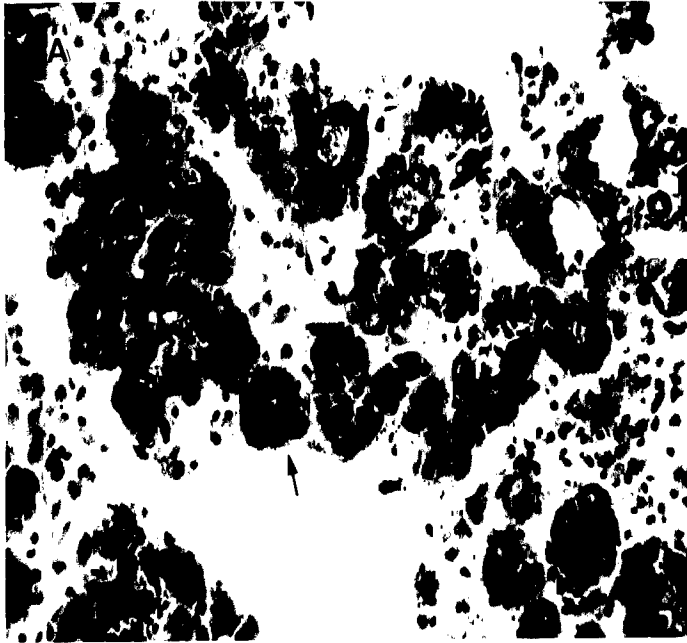
Figure 4.

Immunohistochemistry identifying epithelial cell types in
freshly isolated organoids: (a) shows immunoperoxidase
staining for cytokeratin K19, identifying luminal
epithelial cells (arrow), (b) shows immunoperoxidase
staining for cytokeratin 14, identifying myoepithelial
cells (arrow). All magnifications are 40x.

REPLY TO
ATTENTION OF:

MCMR-RMI-S   (70-1y)   ⊾1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has
reexamined the need for the limitation assigned to technical
reports. Request the limited distribution statement for reports
on the enclosed list be changed to "Approved for public release;
distribution unlimited." These reports should be released to the
National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at
DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl   PHYLIS M. RINEHART
                                        Deputy Chief of Staff for
                                            Information Management

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